Bioremediation of 2,4,6-trinitrotoluene by bacterial nitroreductase-expressing transgenic aspen

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Abstract

Trees belonging to the genus *Populus* are often used for phytoremediation (dendroremediation) due to their deep root formation, fast growth and high transpiration rates. Here, we study the capacity of transgenic hybrid aspen (*Populus tremula x tremuloides* var. Etropole) which express the bacterial nitroreductase, PnrA, to tolerate and take-up greater amounts of the toxic and recalcitrant explosive, 2,4,6-trinitrotoluene (TNT) from contaminated liquid media and soil. Transgenic aspen tolerate up to 57 mg TNT/L in hydroponic media and more than 1000 mg TNT/kg soil while the parental aspen could not endure in hydroponic culture with more than 11 mg TNT/L or soil with more than 500 mg TNT/kg. Likewise, the phytotoxicological limit for transgenic plants to a constant concentration of TNT was 20 mg TNT/L while wild type plants only tolerated 10 mg TNT/L. Transgenic plants also showed improved uptake of TNT over wild type plants when the original TNT concentration was above 35 mg TNT/L in liquid media or 750 mg TNT/kg in soil. Assays with ^{13}C-labeled TNT show rapid adsorption of TNT to the root surface followed by a slower entrance rate into the plant. Most of the ^{13}C- carbon from the labeled TNT taken up by the plant (>95%) remains in the root with little translocation to the stem. Altogether, transgenic aspen expressing PnrA are highly interesting for phytoremediation applications on contaminated soil and underground aquifers.

Introduction

Improper handling, production, storage and decommissioning of the polynitroaromatic explosive 2,4,6-trinitrotoluene (TNT) has led to extensive contamination of soil and groundwater (*1-3*). This xenobiotic is toxic to humans, animals, plants and microorganisms and is recalcitrant to degradation (*1, 3*). To clean up contaminated sites, phytoremediation with plants is receiving increased interest (*4, 5*). For effective phytoremediation, one should make use of plant species that tolerate contaminants, and have the ability to remove large amounts of target chemicals. The use of tree species for phytoremediation (dendroremediation) has several advantages over smaller plants such as large biomass, long life cycle, low nutrient requirements and intrinsic resistance to many pollutants (*6*). Trees belonging to the genus *Populus* are especially useful for phytoremediation because of their deep extensive root system, high water uptake, and rapid growth (*5, 8*). This genus has also successfully been engineered genetically for phytoremediation purposes (*9, 10*). Moreover, poplar trees have been shown to resist up to 5 mg TNT/L and remove this xenobiotic from the medium (*11-14*).

In general, plants appear to deal with TNT as a 'green liver', whereby the contaminant is detoxified and sequestered within plant tissues rather than mineralized to carbon dioxide and nitrogen (*15-17*). Detoxification occurs by transforming the chemical, conjugating it to plant metabolites and then sequestering the resulting macromolecules into vacuoles or polymers such as lignin (*18-21*). As a result, in most terrestrial plants TNT and its derivatives accumulate in the roots and to a lesser extent are transported to the stem and

leaves. Similarly in poplar plants, experiments conducted with ^{14}C-TNT showed that about 75% of the radiolabeled carbon remained in roots and lower stems while only about 10% was translocated to the upper stem and leaves (*11, 14*).

TNT transformation by plants consists mainly of the sequential reduction of the nitro side groups of the molecule by the plant's endogenous nitroreductases to hydroxylamine intermediates and then to amino derivatives. In poplar plants, aminodinitrotoluenes and as yet unidentified polar metabolites have been detected (*11*). The latter polar products are probably the result of reduced TNT derivatives conjugated to plant metabolites. Subramanian *et al*. (*22*) determined in *Arabidopsis thaliana* that the conversion of TNT to hydroxylamine derivatives is rate limiting, leading them to suggest that the most promising enzymes for speeding up TNT removal in plants include those, which catalyze this reduction.

Microorganisms have a whole arsenal of nitroreductases, which efficiently reduce the nitro side groups of TNT to different isomers of aminonitrotoluenes. This transformation of TNT to aminonitrotoluenes is additionally interesting because the reduced products can bind irreversibly to clay and organic material in the soil (*23, 24*). Moreover, many of these enzymes also attack other nitroaromatic compounds, which could be present in contaminated sites. Therefore, in order to improve the phytoremediation capability of plants, bacterial nitroreductases could be engineered into plant genomes. This has been performed with success by Hannink *et al.* (*25*) who obtained transgenic tobacco expressing the nitroreductase gene, *nfsI*, from *Enterobacter cloacae*. Similarly, Kurumata *et al*. (*26*) engineered *Arabidopsis*

thaliana plants to express the nitroreductase, *nfsA*, of *Escherichia coli*. In either case, the transgenic plants could resist higher concentrations of TNT as well as take up greater quantities of the xenobiotic from the medium than its wild type counterparts. Here, we improve upon this theme by using a tree species as the target plant. In fact, we describe transgenic aspen (*Populus tremula* × *tremuloides* var. Etropole) which express the gene encoding the nitroreductase PnrA of *Pseudomonas putida* JLR11 (*27*). This nitroreductase reduces TNT to 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) at a very high rate and also exhibits a relatively broad substrate specificity so that it can reduce other nitroaromatic compounds (*28*). The aim of this work was to study the characteristics of these transgenic plants compared to the parent wild type plants in different media contaminated with TNT.

Experimental Section

Chemicals. TNT was obtained from Unión Española de Explosivos (Madrid, Spain) and was more than 99% pure. 2-hydroxylamino-4,6-dinitrotoluene (2HADNT), 4-hydroxylamino-2,6-dinitrotoluene (4HADNT), 2-amino-4,6-dinitrotoluene (2ADNT), and 4-amino-2,6-dinitrotoluene (4ADNT) were obtained from AccuStandard (New Haven, CT, USA). ^{13}C-TNT was synthesized from >98% pure [ring-U-^{13}C] toluene using the method described by Michels and Gottschalk (*29*).

Bacterial strains and culture conditions. *Pseudomonas putida* JLR11, and *Agrobacterium tumefaciens* C58C1 strains were grown routinely at 28 ºC in Luria Broth (*30*). Preparation of chromosomal and plasmidic DNA, digestion

121 with restriction enzymes and electrophoresis were carried out using standard
122 methods (*30, 31*).

123 **pBIpnrA binary plasmid.** As described previously, the nitroreductase
124 encoding gene, *pnrA*, was obtained by amplifying this gene from *P. putida*
125 JLR11 chromosomal DNA by PCR and cloning the resulting product into pUC19
126 to obtain pNAJ (*28*). This plasmid was digested with restriction enzymes *Xba*I
127 and *Sac*I and the resulting 1 kb fragment was ligated into the binary vector
128 pBI121 (Stratagene, Madrid, Spain), also cut with *Xba*I and *Sac*I, to yield
129 pBIpnrA. In this way, *pnrA* was cloned downstream from the cauliflower mosaic
130 virus 35 S promoter.

131 **Plant material.** Stock shoot cultures of *Populus tremula x tremuloides*
132 var. Etropole subcultured at 6-week intervals on MS medium (*32*), were used as
133 a source for explants. Shoot multiplication of both wild type and transgenic
134 explants were carried out according to Couselo and Corredoira (*33*). All cultures
135 were kept in a growth chamber with 16 h of light provided by cool-white
136 fluorescent lamps, a temperature of 25 ºC and a photon flux density of 50-60
137 $\mu mol \cdot m^{-2} \cdot s^{-1}$. *In vitro* rooted plants were subjected to acclimatization in a tunnel
138 in a greenhouse and after approximately 9-10 weeks were used for hydroponic
139 and soil experiments.

140 **Plant transformation and molecular analyses.** Internodal segments of
141 *in vitro* micropropagated aspen shoots were transformed by co-culture with *A.*
142 *tumefaciens* C58C1 bearing pBIpnrA using the method described by Couselo
143 and Corredoira (*33*). To confirm the presence of the *pnrA* genes, PCR and
144 Southern blot hybridization were carried out using standard methods (*30*) using

DNA isolated from leaves of both transgenic and untransformed plants with the DNeasy plant kit (Qiagen). Oligonucleotide primer pairs for *pnrA* were 5'-AGCCAGCTAACTTACCTGC-3' and 5'-CTCATCCTTCGGTCATAGG-3'. Each successfully transformation event constituted a transgenic line. Two-step real-time RT-PCR was used to determine expression of the transgene in different tissues. Total RNA was extracted with the RNeasy plant mini kit (Qiagen) from leaves, stems and roots of *in vitro* rooted plants and treated with RNase-free DNase Set (DNase I; Qiagen). cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) and oligo-dT primers according to the manufacturer's protocol. SYBR-Green based quantitative assays were performed in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Madrid, Spain). 25 µL reactions consisted of 2.5 µL of cDNA from each sample, 1x SYBR Green PCR Supermix (Bio-Rad, Laboratories, Madrid) and oligonucleotide primer pairs for *pnrA*: 5'-TCAAGACGAAGCACTCAAAGCC-3'; 5'-GGCACGTACTGATCGATGCTGC-3', and for 18S 5'-AATTGTTGGTCTTCAACGAGGAA-3'; 5'-AAAGGGCAGGGACGTAGTCAA-3' (*34*). The PCR conditions were: one cycle at 95 °C for 5 min, followed by 50 cycles at 95 °C for 20 sec, 56 ºC for 30 sec, and 72 °C for 30 sec. Each PCR reaction was performed in triplicate together with controls lacking template. The entire assay, including both the RT and real time PCR steps, was repeated two times from two RNA extractions. Data normalization with 18S was performed using the $\Delta\Delta C_T$ method (*35*). The specificity of the amplifications was verified at the end of the PCR run by using melting-curve analysis and by analyzing PCR products with agarose gel electrophoresis.

Hydroponic assays. To determine the concentration of TNT tolerated by wild type and transgenic plants, opaque covered containers containing 2.5 L of Hoagland medium (*36*) and an aeration system were used. To determine the uptake and toxicity of TNT to wild type and transgenic plants, the effect on transpiration was measured in a gravitational hydroponic system consisting of 250 mL graduated cylinders covered with aluminum foil (to protect roots against light) and filled with Hoagland medium. The roots of acclimatized 10-15 cm long plants were rinsed gently and introduced into either containers or cylinders before fastening with foam rubber plugs. The plants were allowed to acclimatize in the growth chamber for 2 days prior to each experiment. Growth chamber conditions consisted of 60% humidity, 14:10h light: dark photoperiod, 24:18 °C day:night temperature. Each experiment was initiated by replacing the Hoagland medium with medium spiked with different concentrations of TNT. The tolerance assay consisted of exposing four plants to Hoagland medium spiked with a range of TNT concentrations from 0 - 57 mg TNT/L and measuring plant length at a regular basis for 28 days and finally dry weights when the experiment had terminated. The concentrations of TNT and its metabolites were measured (see below) regularly as well. For the uptake experiment, three to four plants were exposed to Hoagland medium spiked with 20, 35 or 50 mg TNT/L and the concentrations of TNT measured at regular time intervals as described below for 48 h. In these experiments the TNT half life was calculated using the slope of the curve multiplied by half the value of the original TNT concentration. For the toxicity experiments, the transpiration of four plants was monitored volumetrically for 2 weeks. These plants were exposed to

193 a relatively constant concentration of TNT by replacing the Hoagland medium
194 spiked with 0, 5, 10, 15, 20, 35 or 50 mg TNT/L every two days. In this
195 experiment, the relative transpiration, **RT**, was calculated as described in
196 Equation 1 by which the amount of medium transpired by each plant (in mL) at
197 each time point (**Tr**) was compared to the mean amount (in mL) transpired by
198 plants of the same line but in control uncontaminated medium ($\mathbf{Tr_0}$) at the same
199 time point. For comparison purposes the RT of plants in uncontaminated
200 medium was calculated at each time point (mean RT always equals 1) and the
201 largest standard error ($p < 0.05$) used as upper and lower limits to obtain the
202 grey bars in Figure 4.

203 To determine TNT and its transformation products in plant organs, at
204 least six plants were exposed for 48 h to Hoagland medium saturated with TNT
205 crystals (approximately 113.5 mg TNT/L). After this period the plants were
206 removed, roots separated from the shoots, and each plant part weighed before
207 freezing at –80 ºC. Prior to freezing, roots were washed three times for 5
208 minutes with 20% (v/v) methanol/water to remove TNT or its metabolites which
209 had adsorbed to the root surface, in order to obtain accurate incorporation
210 data/rates and exclude or minimize false-positive results.

211 **Equation 1**

212 $RT=Tr/Tr_0$

213

214 **Soil assays.** To determine the tolerance of wild type and transgenic
215 plants in soil, the roots of three acclimatized 10-15 cm long plants were rinsed
216 gently and placed in plant pots filled with 1800-2000 g soil spiked with different

quantities of powdered TNT. The soil used originated from the Estación Experimental del Zaidín, Granada, Spain, and is a loamy silt soil classified as Humic Haploxerept (*37*) with the following characteristics: 38% sand; 43% silt; and 19% clay, pH 7.9. The organic matter of this soil is 2.1%, and its $CaCO_3$ content is 8%. Plants were watered regularly with tap water and fertilized once-weekly with 1× Hoagland medium. To mitigate possible leaching of TNT or its metabolites in the soil column, watering was limited to the water-holding capacity of the soil (approximately 30%). Plant length was measured regularly, and at the start and end of the experiment the TNT concentration in the soil was determined as below.

Analytical methods. To determine the concentration of TNT and its transformed derivatives in liquid media, 1 mL samples were centrifuged at 13000 $\times$ *g* for 5 min and/or passed through a GHP Acrodisc 0.45 µm syringe filter (VWR International Eurolab S.L., Barcelona, Spain) prior to analyses by high performance liquid chromatography (HPLC). To determine the concentration of TNT and its transformed products in bulk or rhizosphere soil a modified version of the US-EPA 8330 method was used. Soil samples were prepared by collecting 5-20 g of either rhizosphere soil from the entire length of each plant root or bulk soil from each pot. The samples were dried overnight at room temperature and homogenized with a spatula prior to analysis. For analysis, 2 g of soil (≤2 mm grain size) was added to 10 mL acetonitrile and the mixture shaken vigorously overnight. After settling, 0.5 mL supernatant was collected and mixed with 0.5 mL of a $CaCl_2$ solution (5 g/L). The mixture was centrifuged and the supernatant passed through a GHP Acrodisc 0.45 µm

241 syringe filter before analysis by HPLC. To determine TNT and its transformation
242 products in plant organs, the roots of four 10-15 cm long plants were introduced
243 into Hoagland medium saturated with TNT crystals (approximately 113.5 mg
244 TNT/L) for 48 h before freezing. To extract TNT and its metabolites, frozen plant
245 tissues were homogenized with mortar and pestle in liquid nitrogen and then
246 extracted by shaking overnight with acetonitrile at a ratio of 20 mL acetonitrile
247 per gram wet weight of plant material (28 ºC, 200 rpm). Then, the entire mixture
248 was centrifuged at 10,000 $\times$ *g* for 10 min and the supernatant decanted and 15
249 mL of the supernatant was split into equal volumes (7.5 mL) and each dried
250 using a rotary evaporator. Each half was then either resuspended in 1 mL water
251 and left overnight at room temperature or in 1 mL 1N HCl for hydrolysis of
252 potentially formed conjugates and incubated overnight at 85 ºC. The following
253 day 0.1 volume of water was added to the water solution or 0.1 volume of 10N
254 NaOH to the acidic mixture in order to neutralize the solution. Each mixture was
255 then passed through a GHP Acrodisc 0.45 µm syringe filter and analyzed by
256 HPLC.
257 For HPLC, a Hewlett-Packard model 1050 chromatograph equipped with
258 a diode array detector and a reversed phase column (Novapak 5 µm, C8, 150 x
259 3.9 mm, Waters S.A., Barcelona, Spain) was employed. To analyze TNT and its
260 transformed derivatives in liquid medium or from soil samples, each sample was
261 analyzed for 15 minutes in a 25 mM phosphate buffer (pH 7)/ 33% methanol
262 solution at a flow rate of 0.85 mL/min with the detector set at 230 nm. To
263 quantify, samples were compared with pure standards. In order to analyze
264 possible plant metabolites the HPLC program consisted of 25 mM phosphate

buffer pH 7/ 30% MeOH during 15 min 30 seconds, then increased to 60% methanol in 30 seconds, followed by a gradient to 70% methanol over the following 8 minutes. To re-equilibrate, the concentration was lowered in the next 30 seconds to initial conditions and maintained until the end of the method at 37 minutes. The flow rate was 0.85 mL/min.

^{13}C-TNT experiments. To determine the incorporation of ^{13}C-TNT into plant material, plants were placed into 100 mL flasks filled with 60 mL of Hoagland solution containing 35 mg ^{13}C-TNT/ L and fixed with a foam rubber plug (three replicates). Samples of the liquid media were taken at regular intervals to measure TNT concentration with HPLC. Similarly, whole plants were removed at regular intervals for analysis. Root material was washed three times for 5 minutes with 20% methanol/water (v/v) to ensure that any TNT or derived metabolites adsorbed to the root surface were removed. All plant material was dried at 55 ºC for 72 h prior to analysis in an Elemental Analyzer-Isotope Ratio Mass Spectrometer on-line with a Delta Plus XL mass spectrometer. The overall precision of analyses was ± 0.1 ‰ for ^{13}C. The stable isotope composition was reported as δ^{13}C -values per thousandth (‰): $\delta^{13}C=[(({}^{13}C/{}^{12}C)_{sample}-({}^{13}C/{}^{12}C)_{standard})/ ({}^{13}C/{}^{12}C)_{standard}] \times 1000$. The international standard reference used for ^{13}C/^{12}C was Vienna Pee Dee Belemnite (V-PDB), (calcite from a marine fossil from a Cretaceous-age formation in South Carolina). As atmospheric ^{13}C content is unchanged for all plants, all additional ^{13}C detected can only come via absorption of ^{13}C-labelled TNT from the medium into the plant. Therefore, for comparison purposes an absorption index (I_{abs}) was calculated using δ^{13}C values as follows: $I_{abs}=((\delta^{13}C_{sample} - \delta^{13}C_{control})/$

289 $\delta^{13}C_{control}$), whereby the control values were obtained from plants which had not
290 been exposed to ^{13}C-TNT. The quantity of ^{13}C in roots was calculated by
291 assuming that 46% of root dry weight is carbon (*38*) and multiplying by the
292 difference between the % ^{13}C in each sample with the % ^{13}C in control
293 (unexposed) plants.

294 **Statistical analysis.** For statistical analysis, the Excel 2003 program
295 was used as well as GraphPad InStat 3.06. To measure statistical significance
296 between two data sets, one-tailed unpaired t-tests with Welch correction were
297 applied with significant difference given when $p<0.05$. All error calculations
298 represent standard error at 95% confidence.

299

300 **Results and Discussion**

301 Thirty independent *pnrA* transgenic aspen lines were obtained by
302 *Agrobacterium*-mediated transformation. The line with the highest mRNA levels
303 of *pnrA* was selected for use in subsequent experiments. Characterization of
304 the transcription pattern of *pnrA* in each organ of this transgenic line showed
305 more relative cDNA quantities in leaves (2.58 ± 0.25) and stems (2.15 ± 0.27)
306 than in roots (1.0 ± 0.24). Single copy insertion of the transgene was confirmed
307 by Southern-blot analysis.

308 Experiments under hydroponic and soil conditions were performed to
309 compare the tolerance of wild-type and transgenic trees to different
310 concentrations of TNT. In hydroponic assays, the growth and health of wild type
311 plants were drastically affected at concentrations above 11 mg TNT/L (Figure
312 1A, C and D). Although *pnrA* expressing plants were also affected at this

concentration, these plants continued to show growth in 57 mg TNT/L (Figure 1B and C, E). In contaminated soil, the growth of all plants was affected at 250 mg TNT/ kg soil (Figure 2), and in the case of wild type plants, completely inhibited at concentrations greater than 500 mg TNT/ kg soil (Figure 2A, C, and D). However, *pnrA* transgenic aspen could withstand and still grow at 1000 mg TNT/ kg soil (Figure 2B, C, and E). As a result, after 56 days, the dry weight of *pnrA* transgenic aspen tended to be higher at concentrations of 500 mg TNT/kg and above, and significantly higher ($p<0.05$) at 1000 mg TNT/ kg soil, compared to wild type plants (Figure 2C). Altogether, the results show that *pnrA* expression increases aspen tolerance towards higher TNT concentrations in both hydroponic media and soil.

Thompson *et al*. (*12*) determined that larger, more mature poplar plants could resist higher concentrations of TNT. To test this observation, hydroponic assays were performed using aspen with approximately twice the age of the plants described in the assays above. After 28 days, both wild type and transgenic plants could grow (1.53 ± 1.01 and 3.5 ±1.07 cm, respectively ($n\geq4$)) in 68 mg TNT/L, a concentration prohibitive for younger plants. *pnrA* expressing aspen continued to show growth (4.17 ± 0.61 cm) even at 91 mg TNT/L, while wild type plants, though still alive, did not (0.3 ± 0.11 cm). This has important implications for *in situ* phytoremediation as it implies that soils contaminated with higher concentrations of TNT than reported here could be remediated by transgenic lines if more mature plants are used.

The amount and rate of TNT uptake was measured as well for transgenic and wild type aspen. In the hydroponic experiment to determine the resistance

of each type of plant to TNT (Figure 1), TNT was undetectable within 4 days in all concentrations (data not shown). Depending on the initial TNT concentration, the amount of aminodinitrotoluenes (ADNTs) peaked at 7 days with 1.6 to 4.4 mg ADNT/L (4ADNT and 2ADNT) to slowly decrease to between 0 and 2.8 mg ADNT/L after 28 days. Other TNT metabolites such as hydroxylaminodinitrotoluenes (HADNTs), azoxynitrotoluenes or recently described diarylhydroxylamines or diarylamines (*39*) were not detected in the medium. To study TNT removal in detail, initial TNT concentrations of 20, 35 or 50 mg TNT/L were used (Figure 3A). In the absence of plants, the concentration of TNT changed little throughout the experiment. When exposed to 20 or 35 mg TNT/L, TNT was taken up just as rapidly by wild type plants as by transgenic plants (TNT half life of approximately 30 or 20 h, for 20 and 35 mg TNT/L respectively). However, when exposed to 50 mg TNT/L, *pnrA* transgenic aspen took up TNT more quickly (TNT half life of approx. 30h) than wild type plants (TNT half life of approx. 50 h). In these experiments only negligible amounts of TNT transformation products could be detected. The slower TNT uptake by wild type plants could be due to TNT toxicity. If so, this suggests that the expression of *pnrA* helps the plant to maintain high TNT uptake under otherwise restrictive conditions.

TNT removal was also determined in rhizosphere soil after 56 days (Figure 3B). Wild type plants appear to remove TNT efficiently in soil with low levels of contamination, but at high concentrations (750 mg TNT/kg soil and above) these plants removed less than 50% of the original amount of TNT. On the other hand, consistently more than 60% of the original amount of TNT

disappeared from the rhizosphere soil of *pnrA* transgenic aspen. As a result, TNT removal by *pnrA* expressing plants became more important as the concentration of TNT in the soil increased (≥ 750 mg TNT/ kg soil). However, rather high values were obtained for unplanted bulk soil which remained below those obtained with *pnrA* expressing plants but not always ($p<0.05$) significantly so. Possibly, the low amount of TNT detected in unplanted soils is related to irreversible binding between it and/or its degradation products with humic acids and soil organic and inorganic material (*23, 24*). This also causes a reduction in the bioavailability of TNT for plants especially in aged soils (*11, 12*). This is a major concern for effective phytoremediation but can be partially alleviated by using long-lived trees to ensure a more continuous removal of unbound TNT as it is leached from soil by irrigation. With respect to the presence of TNT transformation products in soil, only ADNTs comprising of 2ADNT (about 33% w/w of the total amount of ADNTs detected) and 4ADNT (about 66%) were detected in either rhizosphere or unplanted bulk soil. The quantity of ADNTs detected in unplanted bulk soil (up to 1.6 mg ADNT/kg soil) was below that measured in the rhizosphere of aspen. The level of ADNTs tended to be higher in the rhizosphere of wild type plants (up to 4.8 mg ADNT/kg soil) than of *pnrA* expressing plants (up to 3.6 mg ADNT/ kg soil). If these differences are due to plant uptake or the action of rhizospheric microbial communities remains unclear. More importantly, the results from both hydroponic and soil experiments demonstrate the greater capacity of transgenic aspen to remove TNT from highly contaminated media. This is in agreement with results obtained with bacterial nitroreductase expressing transgenic tobacco, which also

385 removed significantly larger quantities of TNT from liquid media (*25*) and soil
386 (*40*) than wild type plants.

387 To determine differences in the phytotoxicological limits between wild
388 type aspen and transgenic plants, transpiration was monitored in hydroponic
389 medium in which the concentration of TNT was kept constant. Transpiration
390 rates were used as a parameter because this is more sensitive for determining
391 toxicity in plants than measuring growth or increases in dry weight (*12*). Our
392 results show that wild-type aspen failed to tolerate a constant concentration of
393 TNT above 10 mg TNT/L (Figure 4A), whereas *pnrA*-expressing aspen were
394 only affected at constant concentrations above 20 mg TNT/L (Figure 4B).
395 Thompson *et al.* (*12*) showed that TNT exerted phytotoxicological effects on
396 poplar cuttings at 5 mg TNT/L. The wild type aspen used in this work appear to
397 withstand double this amount of TNT and *pnrA* expression in these trees
398 improves resistance by another twofold.

399 An important concern for phytoremediation of TNT is the fate of the
400 contaminant once it is taken up by the plant. Extraction of roots or shoots of
401 plants used for the above-mentioned experiments did not reveal any clear
402 identification of TNT, transformation products, or products upon hydrolysis of
403 potential conjugates. This indicates that TNT is quickly transformed and
404 sequestered by aspen. Only when plants were exposed for 2 days to liquid
405 medium saturated with TNT was TNT, 4HADNT, 4ADNT and traces of 2ADNT
406 detected in roots (Figure 5) but none in shoots. Azoxynitrotoluenes,
407 diarylhydroxylamines or diarylamines were not detected in any tissue. The
408 concentration of HADNT was higher and of TNT lower in the roots of transgenic

409 plants than in wild type plants. This could be indicative of increased reduction
410 due to *pnrA* expression, but these differences were not statistically significant
411 ($p<0.05$, Figure 5, untreated samples). Although conjugated TNT derivatives
412 have been described in *Arabidopsis thaliana, Catharanthus roseus,* and
413 tobacco (*18, 20, 22*), the only indication for their presence in *Populus* has been
414 as unknown polar transformation products (*11*). In our study, polar peaks, which
415 disappeared upon acid hydrolysis (which could indicate putative conjugates),
416 were not detected in the HPLC chromatograms of either wild type or transgenic
417 root extracts. Moreover, when the amount of TNT together with the transformed
418 products detected in either hydrolyzed or unhydrolyzed extracts are summed
419 up, their total values are similar (Figure 5) indicating that little conjugate
420 formation had occurred. As the plants do not tolerate liquid media saturated with
421 TNT (transpiration stopped within 24 h), possibly the metabolic activity required
422 for conjugate formation was compromised as well. This suggests that the
423 conditions need to be optimized to study conjugate formation and
424 characterization in aspen in greater detail however this is not within the scope of
425 the present work. Comparison with the products detected in other transgenic
426 plants expressing bacterial nitroreductases revealed different tendencies. In
427 *Arabidopsis*, more ADNT but less TNT was observed in *nfsA* expressing
428 transgenic plants than in wild type plants but possible conjugates were not
429 studied (*26*). In wild type tobacco, TNT and ADNT appeared mostly in roots and
430 only very little in shoots. However, no TNT and little ADNT were detected in the
431 roots and shoots of *nfsI* transgenic tobacco plants (*25*). More recently, TNT
432 transformation studies after short (10h) exposures with seedlings of the same

433 transgenic tobacco variant revealed the presence of 4HADNT and greater
434 amounts of conjugates related to this metabolite than in wild type roots
435 indicating enhanced detoxification by the transgenic line (*41*).

436 To obtain information of the distribution of TNT in tissues of either
437 transgenic or wild type plants, ^{13}C ring labeled TNT was used. The experiment
438 was performed with only 35 mg/L of labeled TNT to diminish the possible toxic
439 effect on absorption by plants while maintaining concentrations high enough for
440 detection of the stable isotope, ^{13}C. As expected, ^{13}C enrichment was observed
441 principally in roots with only very little in the shoot compartment (2-6%) (Figure
442 6A). Moreover, similar to TNT uptake results in liquid medium (Figure 3), no
443 significant differences were observed in the absorption of carbon from TNT by
444 wild type or transgenic aspen nor in the disappearance of TNT or its products
445 (principally 4ADNT) in the medium (Figure 6B). Data in Figure 6B shows that
446 the speed of TNT disappearance from the medium is relatively fast, with nearly
447 100% of the initial TNT having disappeared from the hydroponic culture after 24
448 h. However, this contrasts with the rather slow absorption of carbon from TNT
449 into the roots which accounts for approximately 30% of the initial amount of TNT
450 after 4 days. This suggests that adsorption of TNT to the root surface is a rapid
451 process compared to the much slower uptake of TNT into the plant tissue. As a
452 result, probably more TNT would be taken up into the roots if plants had been
453 left for a longer period of time.

454 Taken together, the data presented in our work suggest that the
455 expression of the bacterial nitroreductase *pnrA*, improved the natural capacity of
456 aspen to tolerate, grow, and more importantly, eliminate TNT not only from

457 contaminated hydroponic medium but also from contaminated soil where
458 bioavailability is reduced. The lower expression of the transgene in the root
459 versus the plant shoots suggests that the remediation capacity of *pnrA*
460 expressing aspen could still be improved if greater expression of the transgene
461 could be obtained in roots. Nevertheless, the advantageous characteristics of
462 *pnrA* expressing aspen described in this work make these trees potentially
463 excellent candidates for *in situ* TNT dendroremediation and serve as a proof of
464 concept for the manipulation of other tree species better adapted to other
465 climates and soil conditions.
466
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478 **Literature Cited**

(1) Hawari, J.; Beaudet, S.; Halasz, A.; Thiboutot, S.; and Ampleman, G. Microbial degradation of explosives: biotransformation versus mineralization. *Appl Microbiol Biotechnol* **2000**, *54*, 605-618.

(2) Spain J.C. Introduction. In *Biodegradation of Nitroaromatic Compounds and Explosives.* Spain, J.C.; Hughes, J.B.; Knackmuss, H.J., Ed.; pp 1-5; Lewis Publishers: Boca Raton, 2000.

(3) Smets, B.F., Yin, H., and Esteve-Nuñez, A. TNT biotransformation: when chemistry confronts mineralization. *Appl Microbiol Biotechnol* **2007**, *76*, 267-277.

(4) Singh, O.V.; Jain, R.K. Phytoremediation of toxic aromatic pollutants from soil. *Appl. Microbiol. Biotechnol*. **2003**, *63*, 128-135.

(5) Susarla S.; Medina V.F.; McCutcheon, S.C. Phytoremediation: An ecological solution to organic chemical contamination. *Ecolog. Engin*. **2002**, *18*, 647-658.

(6) Schoenmuth, B. W.; Pestemer, W. Dendroremediation of trinitrotoluene (TNT) Part 1: Literature overview and research concept. *Environ. Sci. Pollut. Res.* **2004**, *11*, 273-278.

(7) Schnoor, J.L.; Licht, L.A.; McCutcheon, S.C.; Wolfe, N.E.; Carreira, L.H. Phytoremediation of organic and nutrient contaminants. *Environ. Sci Technol*. **1995**, *29*, 318-323.

(8) Cherian, S.; Oliveira, M.M. Transgenic plants in phytoremediation: recent advances and new possibilities. *Environ. Sci. Technol.* **2005**, *39*, 9377-9390.

(9) Rugh, C.L.; Senecoff, J.F.; Meagher, R.B; Merkle, S.A. Development of transgenic yellow poplar for mercury phytoremediation. *Nat. Biotechnol.* **1998**, *16*, 925-928.

(10) Doty, S.L.; James, C.A.; Moore, A.L.; Vajzovic, A.; Singleton, G.L.; Ma, C.; Khan, Z.; Xin, G.; Kang, J.W.; Park, J.Y.; Meilan, R.; Strauss, S.H.; Wilkerson, J.; Farin, F.; Strand, S.E. Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16818-16821.

(11) Thompson, P.L.; Ramer, L.A.; Schnoor, J.L. Uptake and transformation of TNT by hybrid poplar trees. *Environ. Sci. Technol*. **1998**, *32*, 975-980.

(12) Thompson, P.L.; Ramer, L.A.; Guffey, A.P.; Schnoor, J.L. Decreased transpiration in poplar trees exposed to 2,4,6 trinitrotoluene. *Environ. Toxicol. Chem*. **1998**, *17*, 902-906.

(13) Ouyang, Y.; Shinde, D.; Ma Q. Simulation of phytoremediation of a TNT-contaminated soil using the CTSPAC model. *J. Environ. Qual.* **2005**, *34,* 1490-1496.

(14) Yoon, J.M.; van Aken, B.; Schnoor, J.L. Leaching of contaminated leaves following uptake and phytoremediation of RDX, HMX, and TNT by poplar. *Int. J. Phytoremediat*. **2006**, *8*, 81-94.

(15) Hughes, J.B.; Shanks, J.; Vanderford, M.; Lauritzen, J.; Bhadra, R. Transformation of TNT by aquatic plants and plant tissue cultures. *Environ. Sci. Technol*. **1997**, *31,* 266-271.

(16) Burken, J.G.; Shanks, J.V.; Thompson, P.L. Phytoremediation and plant metabolism of explosives and nitroaromatic compounds. In

Biodegradation of Nitroaromatic Compounds and Explosives. Spain, J.C.; Hughes, J.B.; Knackmuss, H.J., Ed.; pp 239-275; Lewin Publishers: Boca Raton, 2000.

(17) Hannink, N.K.; Rosser, S.J.; Bruce, N.C. Phytoremediation of explosives. *Cri. Rev. Plant Sci*. **2002**, *21,* 511-538.

(18) Bhadra, R.; Wayment, D.G.; Hughes, J.B.; Shanks, J.V. Confirmation of conjugation processes during TNT metabolism by axenic plant roots. *Environ. Sci. Technol*. **1999**, *33,* 446-452.

(19) Wang, C.; Lyon, D.Y.; Hughes, J.B.; Bennet, G.N. Role of hydroxylamine intermediates in the phytotransformation of 2,4,6-trinitrotoluene by *Myriophylum aquaticum***.** *Environ. Sci. Technol.* **2003**, *37,* 3595-3600.

(20) Vila, M.; Pascal-Lorber, S.; Rathahno, E.; Canlet, C.; Laurent, F. Metabolism of [14-C]-2,4,6-trinitrotoluene in tobacco cell suspension cultures. *Environ. Sci. Technol*. **2005**, *39*: 663-672.

(21) Ekman, D.R.; Lorenz, W.W.; Przybyla, A.E.; Wolfe, N.L.; Dean, J.F. SAGE analysis of transcriptome responses in *Arabidopsis* roots exposed to 2,4,6-trinitrotoluene. *Plant Physiol.* **2003**, *133*: 1397-1406.

(22) Subramanian**,** M.; Oliver, D.J.; Shanks, J.V. TNT phytotransformation pathway characteristics in *Arabidopsis*: Role of aromatic hydroxylamines. *Biotechnol. Prog.* **2006**, *22*, 208-216.

(23) Weiss, M.; Geyer, R.; Russow, R.; Richnow, H.H.; Kästner, M. Fate and metabolism of [15N] 2,4,6-trinitrotoluene in soil. *Environ Toxicol Chem*. **2004**, *23*: 1852-60.

549 (24) Heiss, G.; Knackmuss, H.J. Bioelimination of trinitroaromatic compounds:
550 immobilization versus mineralization. *Curr Opin Microbiol*. **2002**, *5,* 282-
551 287.
552 (25) Hannink, N.; Rosser, S.J.; French, C.E.; Basran, A.; Murray, J.A.H.;
553 Nicklin, S.; Bruce, N.C. Phytodetoxification of TNT by transgenic plants
554 expressing a bacterial nitroreductase. *Nat. Biotech*. **2001**, *19*, 1168-1172.
555 (26) Kurumata, M.; Takahashi, M.; Sakamoto, A.; Ramos, J.L.; Nepovim, A.;
556 Vanek, T.; Hirata, T.; Morikawa, H. Tolerance to, and uptake and
557 degradation of 2,4,6-trinitrotoluene (TNT) are enhanced by the
558 expression of a bacterial nitroreductase gene in *Arabidopsis thaliana*. *Z.*
559 *Naturforsch*. *C* **2005**, *60,* 272-278.
560 (27) Esteve-Nuñez, A.; Ramos, J.L. Metabolism of 2,4,6 trinitrotoluene by
561 *Pseudomonas* sp. JLR11. *Environ. Sci. Technol*. **1998**, *32*: 3802-3808.
562 (28) Caballero, A.; Lázaro, J.J.; Ramos, J.L.; Esteve-Nuñez, A. PnrA, a new
563 nitroreductase-family enzyme in the TNT-degrading strain *Pseudomonas*
564 *putida* JLR11. *Env. Microbiol*. **2005**, *7,* 1211-1219.
565 (29) Michels, J.; Gottschalk, G. Inhibition of the lignin peroxidase of
566 *Phanerochaete chrysosporium* by hydroxylamino-dinitrotoluene, an early
567 intermediate in the degradation of 2,4,6-trinitrotoluene. *Appl. Environ,*
568 *Microbiol.* **1994**, *60*, 187-194.
569 (30) Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory*
570 *Manual*, 2nd ed.; Cold Spring Harbor: Laboratory Cold Spring Harbor, NY,
571 1989.

(31) Ausubel, F.M.; Brent, R.; Kingston, P.E.; Moore, D.M.; Seidman, J.G.; Smith, J.A.; Struhl, K. *Current Protocols in Molecular Biology*. Greene Publishing Associated: New York, N.Y., 1991.

(32) Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **1962**, *15,* 473-497.

(33) Couselo, J.L.; Corredoira, E. Transformación genética de *Populus tremula x tremuloides* con la secuencia *AtPCS1* para su uso en programas de fitorremediación. *Revista Real Academia Galega de Ciencias* **2004**, *13*, 29-94.

(34) Brunner, A.M.; Yakovlev, I.A.; Strauss, S. Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol.* **2004**, *4*, 14.

(35) Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods* **2001**, *25*, 402-408.

(36) Lindsey, K. (Ed). *Plant Tissue Culture Manual; Fundamentals and Applications*. Kluwer Academic Publishers: Dordrecht, 1992.

(37) Soil Survey Staff. *Keys to soil taxonomy*, 9th ed.; United States Department of Agriculture, National Resources Conservation Service: Washington, D.C., 2003.

(38) Hancock, J.E.; Loya, W.M.; Giardina, C.P.; Li, L.; Chiang, V.L.; Pregitzer, K.S. Plant growth, biomass partitioning and soil carbn formation in response to altered lignin biosynthesis in Populus tremuloides. *New Phytol.* **2007,** *273*, 732-742.

(39) Wittich, R.-M.; Haïdour, A.; van Dillewijn, P.; Ramos, J.L. OYE flavoprotein reductases initiate the condensation of TNT-derived intermediates to secondary diaryl amines and nitrite. *Env. Sci. Technol.* **2008**, *42*, 734-739.

(40) Travis, E.R.; Hannink, N.K.; van der Gast, C.J.; Thompson, I.P.; Rosser, S.J.; Bruce, N.C. Impact of transgenic tobacco on trinitrotoluene (TNT) contaminated soil community. *Environ. Sci. Technol.* **2007**, *41*, 5854-5861.

(41) Hannink, N.K.; Subramanian, M.; Rosser, S.J.; Basran, A.; Murray, J.A.H.; Shanks, J.V., Bruce, N.C Enhanced transformation of TNT by tobacco plants expressing a bacterial nitroreductase. *Intern. J. Phytorem.* **2007**, *9*, 385-401.

Figure Legends

FIGURE 1. Comparison of aspen grown in hydroponic media with different TNT concentrations for 28 days. Photographs of (A) wild type aspen and (B) *pnrA* expressing aspen. C) Shoot dry weights and lengths of (D) wild-type and transgenic (E) plants in hydroponic medium growing in hydroponic medium with different TNT concentrations after 28 days. All standard error bars at $p<0.05$, n=4.

FIGURE 2. Growth of aspen in soil contaminated with different concentrations of TNT after 56 days. Photographs of (A) wild-type and (B) *pnrA* expressing aspen. (C) Shoot dry weight and lengths of (D) wild type and (E) *pnrA* expressing aspen in soil after 56 days. For all graphs standard error bars are given at a confidence interval of $p<0.05$ of three repetitions (n=3).

FIGURE 3. Removal of TNT (uptake) by wild-type and *pnrA* expressing aspen from liquid medium and soil. A) Uptake of TNT by wild-type and *pnrA* transgenic aspen exposed to hydroponic medium containing 20 mg TNT/L, 35 mg TNT/L and 50 mg TNT/L (n=4). Dotted lines indicate controls without plants. B) Percentage of original TNT (at T=0) removed in unplanted soil or soil with wild type or *pnrA* expressing aspen (n=3). For all graphs standard error bars are given at a confidence interval of $p<0.05$.

FIGURE 4. Determination of the phytotoxicological limits of (A) wild type and (B) *pnrA* transgenic aspen. Plants were acclimatized for two days (-2 days measurement) and then exposed at T=0 to different concentrations of TNT (mg/L) (standard error bars $p<0.05$, $n\geq3$). Grey bar indicates largest standard error range of the control (RT of plants in medium without TNT). Those concentrations of TNT at which the RT values were below the control (grey bar) at the end of the experiment were considered toxic for the plant.

FIGURE 5. TNT and its transformation products measured in root extracts of wild type plants (wt) or transgenic plants (*pnrA*) which had either been untreated or hydrolyzed (standard error bars $p<0.05$, $n\geq6$).

FIGURE 6. A) Distribution pattern of ^{13}C enrichment from labeled TNT in roots and shoots of transgenic and wild type aspen. Plants were incubated with ^{13}C-TNT and at the indicated times removed and treated as described in the Experimental Section. The $\delta^{13}C$ values were determined from roots and shoots by mass spectrometry and compared using an absorption index to the natural $\delta^{13}C$ levels present in tissues of control plants. B) Adsorption and the subsequent incorporation of ^{13}C from labeled TNT in the root of wild type and transgenic aspen. The percent (w/w) of the initial amount of ^{13}C-TNT present in the flask was determined by measuring the amount of ^{13}C-TNT in liquid media (closed symbols) by HPLC and the quantity of ^{13}C from labeled TNT incorporated into roots

(open symbols) as described in the Experimental Section at different time points. All error bars represent standard error ($p<0.05$) for three repetitions (n=3).